

# Regulatory factor-X binding to mutant HLA-DRA promoter sequences

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## ABSTRACT

The class II genes of the major histocompatibility complex (MHC) encode a family of cell surface glycoproteins that present processed antigen to the T cell receptor. Class II genes are regulated coordinately, responding to both immunologic and developmental signals. Conserved sequence elements 5' to class II genes have been shown to be important in transcriptional control. One of these sequences, the X box, is a specific target for the binding of the factor RF-X. In the hereditary HLA class II deficiency, a form of primary immunodeficiency, a regulatory defect in expression of class II genes is associated with a defect in the binding of RF-X. To determine the basepairs that are important for this binding interaction, a series of single basepair substitutions spanning the X box motif of the DRA gene was constructed and tested for binding of RF-X by gel electrophoresis mobility shift assays (EMSAs). Several, but not all, of the mutants severely affected binding of RF-X. In addition, the binding of both the natural and the recombinant form of RF-X was affected with the same specificity. A comparison of X box basepair positions important for RF-X binding to DRA with sequences conserved between X boxes of other class II alpha chain genes suggests that high affinity RF-X binding is important for a high level of expression and may explain differences in the levels of class II expression of different class II alpha chains.

## INTRODUCTION

The class II genes of the major histocompatibility complex (MHC) encode a set of heterodimeric, cell surface glycoproteins responsible for the presentation of processed antigen to CD4-positive T lymphocytes. This interaction facilitates both the initiation of a specific acquired immune response (1,2), as well as the education of immature T cells in the thymus (3). Due to the highly specific nature of these two processes, transcription of these genes is tightly controlled on several levels. Class II genes are primarily expressed in a tissue-specific manner, restricted to mature B lymphocytes, activated T cells, dendritic cells,

macrophages, and thymic epithelial cells. Expression in the B lymphocyte lineage is further subject to developmental control as class II-negative pre-B cells require a variety of inducers for class II activation (4,5). Finally, gamma-interferon can induce class II gene expression in non-lymphoid cells, e.g., fibroblasts and endothelial cells (6). Aberrant regulation of class II genes is associated with certain disease states in humans. Class II expression in normally silent tissues has been associated with some autoimmune disorders (7). In contrast, the lack of class II gene expression characterizes a type of primary immunodeficiency that is due to a defect in HLA class II gene regulation (reviewed in(8)).

The human MHC class II proteins or HLA antigens are coordinately expressed from a multigene family in the HLA-D region, subdivided as DR, DQ, and DP. Both alpha and beta chain components of the class II molecule are encoded by each subregion. All class II genes share a set of conserved upstream sequences (CUS) in their promoter regions that can be divided into several discrete elements. These include the X box, the Y box, and the spacer sequence separating the two motifs. In addition, a region upstream of the X box contains a conserved heptamer motif that has been termed the W box or the Z box. DNA-mediated transfection studies have shown that 160 bp of DNA sequence upstream of the DRA and DQB transcriptional start sites were necessary and sufficient for both constitutive and inducible expression while fine deletion analyses and, more recently, linker scanning mutagenesis studies assigned different transcriptional functions to each of the CUS elements (9-18). The X box region is necessary for B cell-specific expression and  $\gamma$ -interferon induced expression, while the Y box functions as a non-B cell specific positive element (10,12,14,19,20). In addition, a transcriptionally defective class II-negative B cell line, RJ2.2.5, failed to use the X box as a positive activating element in transient expression assays, further supporting the role of the X box in class II gene expression (12,19). The W box region responds to gamma-interferon induction in nonlymphoid cells (9,10,16,21).

An array of sequence-specific DNA-binding proteins have been shown to interact with the CUS elements from different class II genes (Reviewed in (22)). Gel electrophoresis mobility shift assays (EMSAs) using extracts from a variety of cell types have

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detected several X box binding factors. Two of the proteins that have been identified that bind within or near the HLA-DRA X box region include RF-X (Regulatory Factor-X) and NF-S (Nuclear Factor-Spacer) (23). RF-X binds to the center of the DRA X box, whereas NF-S binds to a region that includes the last 3 bp of the X box and extends into the 5' side of the spacer region. RF-X shows decreasing affinity for other class II alpha chain X box sequences DPA and DQA, respectively; whereas, NF-S displays the opposite affinity pattern for X box sequences, binding to DQA best and to DRA very poorly. The gene for RF-X has been cloned and the recombinant form was shown to have similar binding characteristics as the RF-X protein in crude B cell extracts (24,25). For the murine I-A $\alpha$  X box, one protein, mXBP, has been identified that binds to the spacer sequence and the 3' side of the X box. It is not known if mXBP and NF-S are homologous proteins. The cloning and analysis of mXBP has shown it to be a cAMP response element binding protein that can dimerize with c-Jun (26,27). Its role in class II expression is still unknown.

The analysis of cell lines from patients with class II deficient combined immunodeficiency (CID) has shed some light on the role of RF-X in class II expression. In these individuals, class II genes are not transcribed, and it was shown that RF-X binding in these cell lines was defective (28). It is now evident that the X box is important for the control of class II gene expression and likely that expression is mediated by the factor RF-X. In this report the DNA binding specificity of cellular RF-X and a recombinant X box protein (rRF-X) was determined by examining the effects of single basepair substitutions in the X box region on binding. The results showed that they were nearly identical and present a profile accurately describing the binding pattern of RF-X.

## MATERIALS AND METHODS

### Cell Culture and Nuclear Extract Preparation

The class II-positive Burkitt's lymphoma cell line Raji was grown in RPMI 1640 medium (GIBCO, Inc.), supplemented with 2% fetal bovine serum, 8% calf serum, 100 U penicillin per ml, 100  $\mu$ g of streptomycin per ml, and 2 mM L-glutamine. Nuclear extracts were prepared from 10 g of packed cells as described by Shapiro *et al.* (29). Fifteen mg of nuclear extract was chromatographically separated over an Affigel heparin agarose column (BioRad, Inc.) as described by Jones *et al.* (30). A 200 ml linear gradient (0.1–0.75 M KCl) was used to elute the proteins off the column into 3 ml fractions. Both crude and fractionated nuclear extracts were stored at  $-70^{\circ}\text{C}$ .

### Recombinant Protein Preparation

The plasmid pRFX9 was constructed by inserting the RFX9 cDNA insert from phage  $\lambda$ 9 (24,25) into the plasmid pT7-7 (31) in frame with the translation initiation codon. Capped mRNA was synthesized *in vitro* from 1  $\mu$ g of linearized pRFX9 by incubation with 10–20 units of T7 RNA polymerase, for 1 hour at  $37^{\circ}\text{C}$  in a 20  $\mu$ l volume containing 40 mM Tris HCl, pH 8.9, 8 mM MgCl<sub>2</sub>, 2 mM spermidine, 50 mM NaCl, 30 mM DTT, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM UTP, 0.05 mM GTP and 0.5 mM m<sup>7</sup>G(5')ppp(5')G. After transcription, the reaction was diluted with water and precipitated with ethanol. The mRNA was dissolved in water and translated in a rabbit reticulocyte lysate system (Promega, Inc.), in the presence of <sup>35</sup>S-methionine, as specified by the manufacturer.

### Probe and Competitor Preparation

Oligonucleotides, DRAX, DQAY, DQBW, were synthesized on a Gene Assembler Plus Oligonucleotide Synthesizer (Pharmacia, Inc.). The sequence of DRAX and DRAY are shown in Figure 1. The sequence of DQBW is

5' GGCAATTGAAAGACGTCCAGTGCCAGGCACTGGATTTCAGAACCTTCACA  
GTTAACCTTCTGCAGGTACGGTCCGTGACCTAAGTCTTGAAGTGTC 5'

Individual strands were purified by denaturing polyacrylamide gel electrophoresis. Complementary strands were boiled for 5 minutes in 0.2 M NaCl, 1 mM MgCl<sub>2</sub>, transferred to a  $65^{\circ}$  heat block, and allowed to cool to room temperature over 2 hours. The double-stranded oligonucleotides were end-labeled using [ $\gamma$ -<sup>32</sup>P]ATP and T4 polynucleotide kinase to a specific activity of approximately  $2 \times 10^8$  cpm/ $\mu$ g. Probes were purified on a polyacrylamide gel, eluted into a solution of 0.5 M ammonium acetate and 1 mM EDTA at  $37^{\circ}\text{C}$  overnight, and concentrated on a Nensorb 20 column (DuPont Inc.).

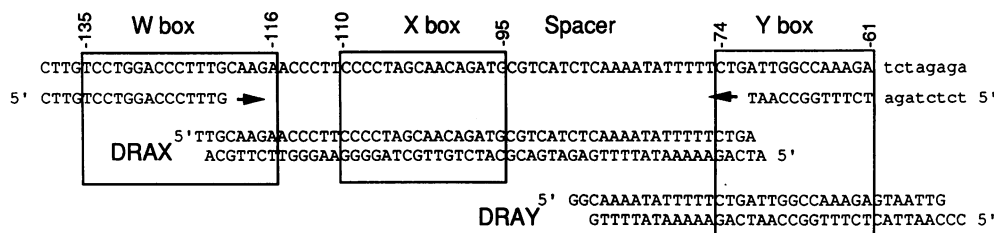
Oligonucleotides containing single basepair substitutions spanning the DRA promoter region were synthesized (Synthecell, Inc.) by contaminating each of the four phosphoramidites with each of the other three as previously described by Hutchison *et al.* (32). Complementary oligonucleotides were annealed, cloned and analyzed by DNA sequencing (The details of these basepair substitutions and others will be published elsewhere). Competitor DNAs containing individual point mutations spanning the X box region were generated by amplification of the mutant sequences using the polymerase chain reaction. The primers for these reactions are described in Figure 1. Amplified DNA was purified on a polyacrylamide gel, eluted into a solution of 0.5 M ammonium acetate and 1 mM EDTA, ethanol precipitated, and resuspended in 0.2 M KCl. Competitor DNA concentrations were determined by optical fluoroscopy (Hoefer Model TKO 100).

### Electrophoretic mobility shift assays (EMSAs)

A 20  $\mu$ l DNA-protein binding assay contained 12 mM HEPES-KOH (pH 7.9), 12% glycerol, 60 mM KCl, 5 mM MgCl<sub>2</sub>, 0.12 mM EDTA, and 0.3 mM DTT (28). Four  $\mu$ l of a Raji nuclear extract fraction (approx. 0.15  $\mu$ g protein) were assayed with 0.2  $\mu$ g of poly (dIdC)-(dIdC) (Pharmacia, Inc.) and 0.05  $\mu$ g of sonicated denatured salmon sperm DNA as nonspecific carrier DNA. Recombinant RF-X (1–2  $\mu$ l) were assayed in the presence of 1.0  $\mu$ g poly (dIdC)-(dIdC) and 0.5  $\mu$ g denatured salmon sperm DNA as carrier.

Protein extracts were preincubated under the above conditions with carrier and competitor DNA on ice for 10 minutes. 20,000 counts per minute of end-labelled DNA probe (approx. 0.1–0.5 ng) were added and the reaction incubated for an additional 30 minutes on ice. Samples were analyzed on a native 4% polyacrylamide gel (acrylamide:bis ratio = 29:1) in 25 mM Tris, 25 mM boric acid, 0.5 mM EDTA. Gels were electrophoresed with buffer recirculation at  $4^{\circ}\text{C}$  for two hours at 200 volts, fixed with a solution of 10% acetic acid and 10% methanol, dried, and exposed to X ray film either overnight with an intensifying screen at  $-70^{\circ}\text{C}$  or for several days without a screen. EMSAs carried out with recombinant RF-X were exposed to two pieces of film to filter out the <sup>35</sup>S label. All competition experiments were carried out at least three times with similar results.

Relative RF-X band intensities on autoradiographs were determined using a video densitometer (BioRad, Inc.; Model 620). Competitive activity of each mutant was expressed as a



**Figure 1:** Oligonucleotide probes and competitors. The DRA conserved upstream sequences (CUS) are delineated by boxes with basepair positions upstream from the major transcriptional start site as determined by Schamböck *et al.* (35). The double-stranded oligonucleotides DRAX and DRAY are illustrated. Oligonucleotide primers used to generate RF-X competitor DNAs by the polymerase chain reaction are indicated with arrowheads denoting their 3' ends. Lowercase DNA sequence represents vector sequences into which the mutated X box DNAs were cloned.

percentage of the change in band intensity seen with 25 ng of mutant competitor relative to the reduction seen with 25 ng of wildtype competitor.

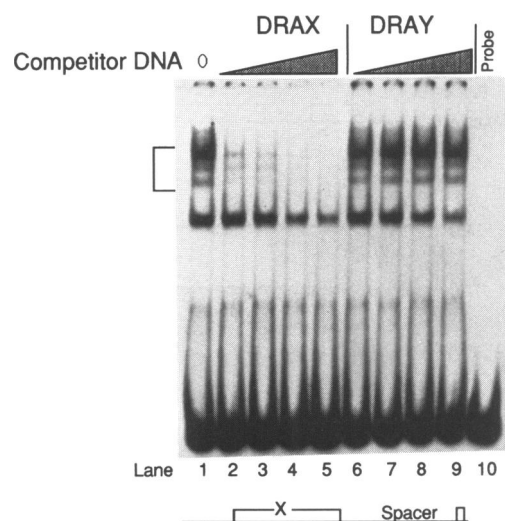
## RESULTS

### Specificity of RF-X for DRA X box sequences

To evaluate individual base pairs important for RF-X binding specificity, X box-specific binding activity was identified in our B cell nuclear protein extracts. EMSAs were performed using a double-stranded oligonucleotide containing the DRA X box sequence as a probe (Figure 1). This probe is identical to the one used by Kober *et al.* (23) to demonstrate RF-X activity in crude B cell nuclear extracts. A crude nuclear extract of Raji cells was fractionated by heparin agarose column chromatography. X box binding activity eluted from the column at about 0.35 M KCl. Binding of the active fraction to the DRA X box oligonucleotide in an EMSA produced several discrete bands (Figure 2A, lane 1). Increasing amounts of the DRA X box oligonucleotide readily competed for the uppermost four complexes (one major and three minor bands) indicating that all four were X box-specific (Figure 2B, lanes 2–5). These results were similar to those obtained by Kober *et al.* (see figure 2 in reference (23)). As a nonspecific control, an oligonucleotide competitor, DRAY, containing the DRA Y box sequence was unable to compete for these complexes at a ten-fold higher concentration of competitor (Figure 2A, compare lanes 2 and 9). Additionally, a competitor DNA encoding the DQB X box sequence weakly competed for these same bands suggesting some cross-activity for RF-X binding between the two X boxes (data not shown).

### Mutant X box sequences as competitors for native RF-X binding

To identify specific bases in the X box region important in RF-X binding, a series of single basepair substitutions was constructed and analyzed for their ability to specifically compete with a labeled DRA X box probe. A series of oligonucleotides containing single point mutations was synthesized by using an oligonucleotide synthesizer in which each of the four nucleotide reagents was contaminated with the other three nucleotides. Mutants were isolated containing substitutions between bases –115 and –86, encompassing regions both upstream and downstream of the DRA X box (–110 through –95) (Figure 3). DNA competitors for EMSAs were generated from clones of each mutant by the polymerase chain reaction. PCR-amplified competitor DNA sequences are as effective as double-stranded



**Figure 2:** RF-X specificity for DRAX in a fractionated Raji nuclear extract.  $^{32}$ P-end-labeled DRAX DNA was used to probe for RF-X binding activity in a Raji nuclear extract fractionated by heparin agarose chromatography. An autoradiograph of an EMSA is shown, using one of the most active fractions obtained from the column. The amount of unlabeled oligonucleotide competitors incubated in the binding reactions was as follows: lane 1, no competitor; lanes 2–5, 0.1, 0.2, 0.5, and 1.0 pmol of DRAX competitor DNA; lanes 6–9, 0.1, 0.2, 0.5, and 1.0 pmol of DRAY competitor DNA. The bracket at the left of the autoradiogram denotes the position of the DRA X box-specific complexes.

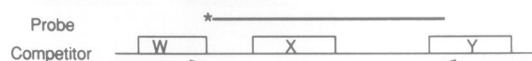
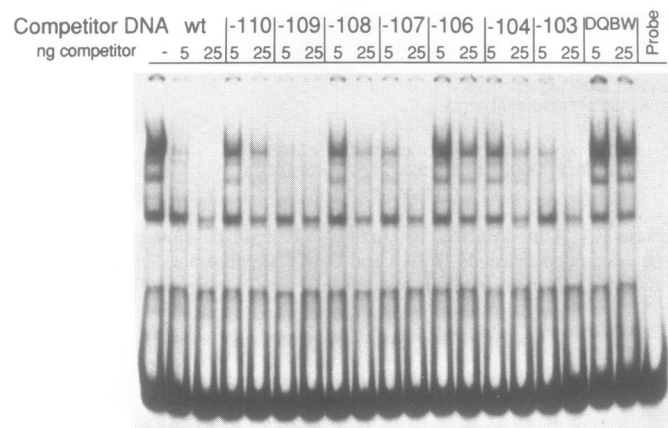


**Figure 3:** Single basepair substitution mutants spanning the DRA X box region. The sequence of the HLA-DRA CUS surrounding the X box is shown with basepair positions numbered from the transcriptional start. Basepair substitutions are indicated below the arrows.

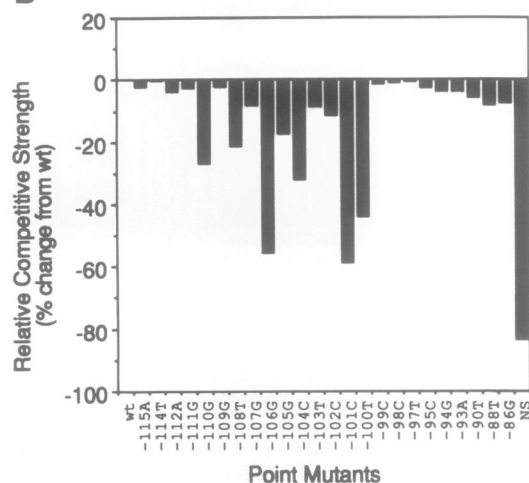
oligonucleotide sequences in their ability to compete for RF-X (data not shown). The PCR-generated competitors also contain sequences for the W and Y boxes, but appear not to alter the results of these competition reactions.

To assess the effect of X box point mutations on binding to RF-X, PCR-generated fragments were used as unlabeled competitors in the EMSA and the results compared to those obtained with the wildtype X box sequence and nonspecific

A



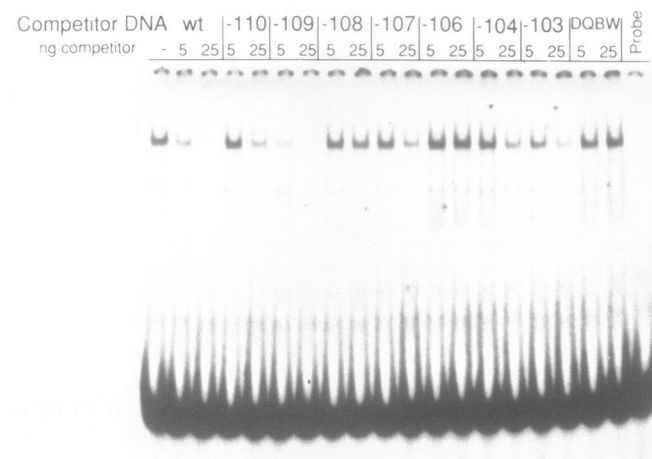
B



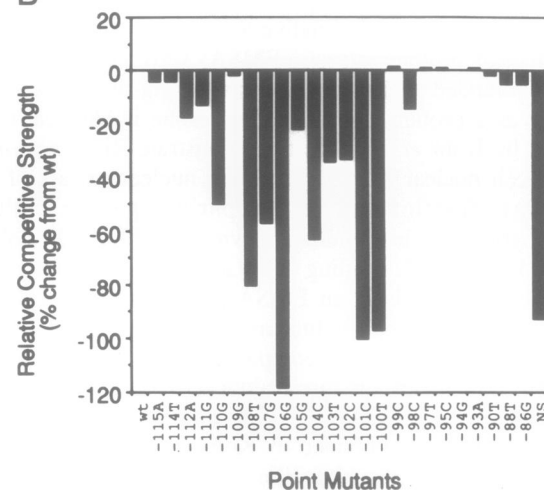
**Figure 4:** Competition for native RF-X by X box point mutants. (A) Autoradiograph of an EMSA using PCR-generated X box point mutants as competitors for RF-X binding. Binding reactions were performed on 0.15  $\mu$ g of fractionated Raji extract by incubating labeled DRAX DNA probe with competitor DNA. Mutant competitor DNA and the amount used in each binding reaction is indicated. (B) Summary of all RF-X competition assays with DRAX X box point mutants using 25 ng of competitor DNA. Individual bands intensities on autoradiographs were determined by densitometry and competitive strength relative to wildtype was calculated as described in the Materials and Methods. Individual mutant competitors are indicated along the horizontal axis. Competitive strength of each mutant, expressed as a percentage change from that of the wildtype competitor, is indicated on the vertical axis.

competitors. Competition experiments with eight of the single point mutants are shown in Figure 4A. The data from all of the mutants isolated were analyzed by densitometry and summarized in Figure 4B. In these graphs, competitive activity is plotted as a percentage of the wildtype competitor activity at 25 ng, an amount in which differences are easily detected. The greatest impairment in competitive activity for RF-X was seen with mutations at -106, -101, and -100, all showing a reduction of competitive activity of over 40% relative to wildtype. A more extensive competition analysis of mutants -106, -101, and -100 demonstrated that 15- to 20-fold more mutant DNA was

A



B

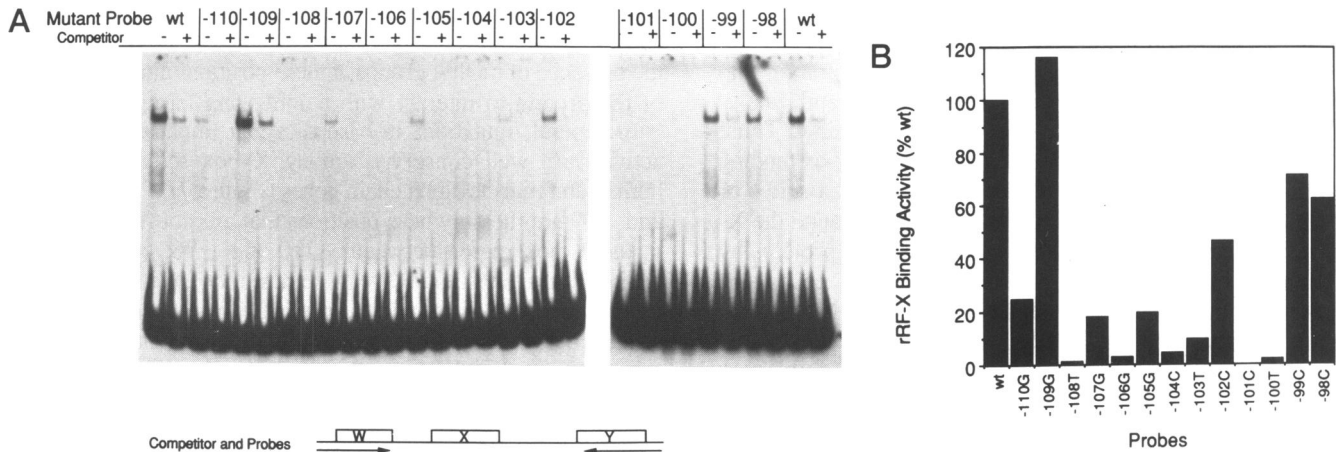


**Figure 5:** Competition for recombinant RF-X by X box point mutants. (A) Autoradiograph of an EMSA using PCR-generated point mutants as competitors for recombinant RF-X (rRF-X). Binding reactions were performed as described above, using 2  $\mu$ l of *in vitro* translated rRF-X per reaction. Identical competitors to those used in Figure 4A are shown. (B) Summary of rRF-X competition assays with all DRAX X box point mutants using 25 ng of competitor DNA. Relative competitive strength of each mutant was determined as a percent change from wildtype levels, as described in Figure 4B.

necessary to compete for RF-X protein than wildtype DNA (data not shown). Mutations at -110, -108, -107, -105, -104, -103, and -102 showed an intermediate level of competitive strength (competitive activity reduced between 8 and 40%) while those at positions -115, -114, -112, -111, -109, and all mutants analyzed from -99 through -86 competed for the X box-specific complexes as strongly as the wildtype competitor. These data center the RF-X binding activity from bases -110 to -100.

#### Mutant X box sequences as competitors for recombinant RF-X

To examine sequences necessary for the binding of RF-X in a system free of other nuclear proteins, X box point mutants were



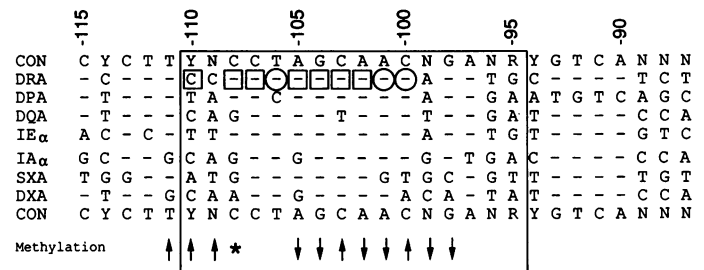
**Figure 6:** X box point mutants as probes for rRF-X. (A) EMSA of rRF-X binding to mutant probes. PCR-amplified X box mutants were  $^{32}\text{P}$  end-labeled to equivalent specific activities and incubated with rRF-X as described above with or without 5 ng of PCR-generated wildtype DNA as a competitor. The base substitutions and presence (+) or absence (-) of wildtype DRA X box competitor DNA is indicated. (B) Summary of rRF-X binding activity to mutant X box probes. Band intensities on the autoradiograph were determined by densitometry, and the binding activity of each point mutant was expressed as a percentage of that seen with the wildtype probe. Base substitutions of each probe are shown on the horizontal axis.

analyzed for their competitive activity for recombinant RF-X (rRF-X) with the DRAX probe. A plasmid containing an N-terminally truncated cDNA fragment of RF-X was transcribed and translated *in vitro*. As shown in Figure 5A, the recombinant protein product shifts the DRAX probe to a discrete band in an EMSA. This complex is disrupted with wildtype PCR-generated DR CUS sequences (wt) as compared to comparable amounts of a nonspecific competitor (DQBW), demonstrating the formation of a DRA X box-specific complex.

DNA competition experiments with eight of the point mutants are shown in Figure 5A, and the data from all of the mutants isolated and analyzed, as described above, are summarized in Figure 5B. Mutations at -106, -101, -100 were unable to compete for rRF-X (competitive activity reduced over 90%) while those at -110, -108, -107, -105, -104, -103, and -102 were strongly impaired in their competitive ability (reductions between 20 and 90%). Mutations at positions -115, -114, -112, -111, and -109 and all point mutants analyzed from -99 through -86, however, competed for rRF-X as strongly as the wildtype competitor. The competition data with rRF-X (Figure 5B) are essentially identical to those obtained with the protein extract from Raji cells (Figure 4B), suggesting that the binding activities in Raji cells and the recombinant protein are the same.

#### Mutant X box sequences as probes for RF-X binding

Competition experiments do not directly address whether a particular X box point mutant can bind RF-X but rather the ability of the mutant DNA to compete with the wildtype DNA sequence for the protein. A sequence that competes poorly may still bind the specific protein with a lowered affinity. To determine if the mutant X box sequences could bind RF-X protein, PCR-generated mutant X box sequences were labeled as probes for direct RF-X binding assays. Recombinant RF-X was used in these assays to avoid interference with other factors that may bind to the W, Y, and spacer regions contained on the PCR-generated probes. The results shown in Figure 6 demonstrated that the ability of the mutant sequences to bind RF-X protein directly correlated with the ability of the mutant sequences to compete for RF-X protein. A C to G substitution at -109 bound more protein than



**Figure 7:** Significance and comparison of human and murine alpha chain gene X box sequences. Comparison of X box sequences from human and murine alpha chain genes. The consensus sequence (CON) is shown at top and bottom. Basepair numbering is according to the DRA sequence as determined by (35). Identity with the consensus is denoted by a dash (-) and base substitutions are as indicated. DPA(23), DQA (33), IEA (15), IAA (17), SXA (36), DXA (33). 'Methylation' shows a summary of methylation interference data for RF-X and rRF-X as previously determined (23,24,28). Both strong and weak interference positions are treated equally. Up and down arrows indicate interference on the 5' and 3' strands, respectively, and a \* indicates a position where methylation enhanced binding. The box encloses the X-box region. In the DRA row, circled bases indicate positions at which substitutions failed to compete for RF-X protein, and boxed bases indicate positions at which substitutions produced a significant effect on the ability of the sequence to compete for RF-X protein.

wildtype, suggesting the creation of a higher-affinity RF-X binding site. Probes containing mutations that showed an impaired level of competitive activity for RF-X were also less effective as probes for RF-X protein and were competed by wildtype competitor DNA. Several mutants barely formed complexes with rRF-X including the five mutants that displayed the greatest decrease in competitive activity: -108, -106, -104, -101, and -100 (Figure 5B).

#### DISCUSSION

The expression of class II MHC genes in B cells has been shown to be controlled by the conserved sequence element, the X box. Using B cell extracts several different proteins have been reported to specifically bind to this region yet it is not clear which of the detected proteins plays a role in class II expression. Additionally,

comparison of the EMSAs from different reports does not lead to a consensus identification of one or a set of proteins. Differences in the assays with regard to extract preparation, the X box sequence (gene and species differences), and finally binding conditions are probable causes of this uncertainty. Thus, it is likely that discrimination between the different X-box binding activities will prove difficult. To address this issue, a series of single basepair substitutions was generated in and around the X-box motif that can be used to identify DNA binding proteins by their ability to interact with the different sequences, i.e., a sort of DNA binding fingerprint. This analysis of DNA-protein interactions differs from methylation-interference studies in that individual basepairs can be assayed for their effects on binding activity, and the magnitude of these effects can be determined. Although not addressed in this paper, a comparison between the binding patterns of the detected proteins and transcriptional activity of the mutant promoters may be the only way to determine which proteins are in fact regulating transcription.

The absence of binding of one of the X box factors, RF-X, has been shown to correlate with the genetically transmitted disease CID and is perhaps the only class II DNA binding protein that has both a genetic and biochemical characterization. In this report, EMSAs were carried out to establish a 'fingerprint' with respect to which sequences affected binding, for the RF-X protein. Additionally the results were compared to a cloned RF-X protein and found to be essentially identical. Thus, a fingerprint for RF-X has been established.

The region bound by RF-X is centered between positions -110 through -100 as mutations in bases 5' and 3' to this region could compete for RF-X binding from a DRA X box probe as strongly as wildtype sequences. Individual bases within the X box region could be further subdivided by their relative competitive activities. Basepair substitutions at -106, -101, and -100 were inactive competitors, implying that these positions are most important for RF-X binding activity. Mutations at positions -110, -108, -107, -105, -104, -103 and -102 showed an impaired ability to compete for these complexes while a substitution at position -109 competed for RF-X binding as strongly as wildtype sequences and is probably not critical for binding activity. The converse experiment, using individual mutants as probes for rRF-X binding, confirmed these relationships. A similar analysis of the recombinant RF-X protein yielded essentially identical results, confirming that the cloned protein is RF-X. Moreover, since the recombinant protein is sufficient to confer X box binding activity, it is likely that no other protein interactions are necessary for the binding of native RF-X to the X box site.

These data are strikingly similar to previously reported methylation interference data for RF-X binding (Figure 7). Previous studies on RF-X (23-25,28) demonstrated strong methylation interference at positions -110, -104, -103, and -100 and weaker interference sites at -111, -109, -107, -105, -102, -101, -99, and -98. In addition, methylation at position -108 appeared to enhance RF-X binding. The methylation interference data was not able to detect position -106 as one important for RF-X binding. The methylation reaction is specific only for protein contact points close to the N-7 position of guanines in the major groove and the N-3 position of adenines in the minor groove. Our results imply that RF-X may contact basepair -106 (normally a T-A basepair) in the major groove or that the transversion at this position may interfere with contacts at positions -105 and or -107. In contrast, methylation at positions -111, -109, and -99 weakly interfered with RF-X binding yet mutations at these sites did not inhibit binding activity.

Perhaps these mutations were tolerated whereas other substitutions would not be tolerated. Another possibility is that the presence of methyl groups at these positions altered the ability of the protein to interact with neighboring basepairs.

In general, mutations in basepairs that impaired competitive activity are well conserved among X box sequences in both human and murine alpha chain genes (Figure 7). Positions -109 and -99 are the only base positions that are not fully conserved in the X box region between -110 and -98, consistent with our result that substitutions at these positions have no detrimental effect upon RF-X binding. Interestingly, the DQA X box sequence diverges from the consensus sequence, having identical substitutions as our DR mutants at -108 and -103. Individually, these mutations greatly reduced binding activity for RF-X. Additionally, Kobr *et al.* (23) found a decreasing order of RF-X affinity among alpha chain X boxes with DRA binding the strongest and DQA sequences binding the weakest. Since DQA is transcribed at a much lower level than DRA, perhaps these two base substitutions in DQA account for this lowered affinity for RF-X, which may in turn, affect the overall transcription rate of the gene. In addition, the X box of DXA diverges from the consensus at positions -108, -105, and -100, all of which impaired binding activity in our assays. In particular, the mutation at -100 most severely affected competitive activity and, when used as a probe, could not form a complex with RF-X at all. Thus, an RF-X binding defect predicted from the DXA X box sequence may account for the lack of detectable transcripts from this gene (33,34).

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